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Toward Reliable Lipoprotein Particle Predictions from NMR Spectra of Human Blood: An Interlaboratory Ring Test

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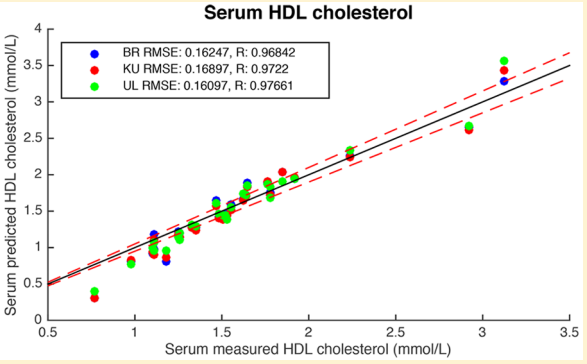
Supporting Information

ABSTRACT: Lipoprotein profiling of human blood by ^{1}H nuclear magnetic resonance (NMR) spectroscopy is a rapid and promising approach to monitor health and disease states in medicine and nutrition. However, lack of standardization of measurement protocols has prevented the use of NMR-based lipoprotein profiling in metastudies. In this study, a standardized NMR measurement protocol was applied in a ring test performed across three different laboratories in Europe on plasma and serum samples from 28 individuals. Data was evaluated in terms of (i) spectral differences, (ii) differences in LPD predictions obtained using an existing prediction model, and (iii) agreement of predictions with cholesterol concentrations in high- and low-density lipoproteins (HDL and LDL) particles measured by standardized clinical assays. ANOVA-simultaneous component analysis (ASCA) of the ring test spectral ensemble that contains methylene and methyl peaks (1.4−0.6 ppm) showed that 97.99% of the variance in the data is related to subject, 1.62% to sample type (serum or plasma), and 0.39% to laboratory. This interlaboratory variation is in fact smaller than the maximum acceptable intralaboratory variation on quality control samples. It is also shown that the reproducibility between laboratories is good enough for the LPD predictions to be exchangeable when the standardized NMR measurement protocol is followed. With the successful implementation of this protocol, which results in reproducible prediction of lipoprotein distributions across laboratories, a step is taken toward bringing NMR more into scope of prognostic and diagnostic biomarkers, reducing the need for less efficient methods such as ultracentrifugation or high-performance liquid chromatography (HPLC).



In the postgenomic era, metabolomics plays a key role in life sciences as the real-time downstream manifestation of the genomic potential of an organism. The numbers of biological studies that involve metabolomics are increasing. Despite being an informative and promising approach, metabolomics is still considered to be a nonstandardized method of analysis. Indeed, methodologies and protocols used in metabolomics of a single organism differ between laboratories and projects, which in turn leads to data incompatibility and a lack of knowledge due to lack of standardization. It is therefore crucial to introduce optimized and standardized metabolomics protocols in order to map the metabolomes of organisms in a reproducible way. Since high-resolution NMR spectroscopy was first applied to human blood,[1] it has become one of the most widely used analytical platforms in metabolomics for studying human blood plasma and serum of large cohorts.[2] Many studies have identified human disease biomarkers[3] and dietary effects on human health using NMR-based metabolomics on blood and urine.[4] Recent developments on standardization and optimization of NMR-based metabolomics protocols for human biofluids further enhanced the throughput and reproducibility of the methods.[5,6] NMR spectroscopy allows simultaneous detection of a wide range (50−70) of structurally diverse

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metabolites from human plasma or serum.[4,7−10] Despite the fact that mass spectrometry (MS)-based methods are more sensitive, with several hundreds of detectable metabolites within a single run, NMR spectroscopy outperforms the MS-based methods in terms of its high reproducibility, speed, and ability to quantify metabolites in an absolute manner without cumbersome calibration procedures.[3] Hence, NMR spectroscopy has established itself as a reliable tool for quantification of the human blood plasma and serum metabolome in a high-throughput manner.[2] The latter fact indeed places NMR as a method of choice for large international cohorts involving thousands of measurements over several years.[2]

NMR has one more overlooked advantage, namely, that it is nondestructive and allows for measurement of a sample as is. This makes NMR a unique platform for investigating lipoproteins since it can assess size and density of these supramolecular aggregates in their intact form. This is a considerable advantage over other omics platforms which typically only consider the molecular composition of biofluids. Lipoprotein particles are supramolecular triglyceride and cholesterol transport vehicles in blood and can be divided into four main groups on the basis of their density and size: very low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL). The lipoprotein particles can be further separated into subgroups and large chylomicrons.[11] Their determination provides valuable information, as they constitute key biomarkers and risk factors of cardiovascular diseases and portray the complex link between obesity and disease.[12,13] Unfortunately, detailed determination of lipoprotein particle distributions (LPDs) relies on time-consuming, complex, and inefficient separation-based methodologies such as ultracentrifugation (UC)[14] or high-performance liquid chromatography (HPLC).[15] Density gradient ultracentrifugation can separate lipoprotein classes within 2−5 days with coefficients of variation (CVs) ranging from 3% to 9% (+4% in the HDL class),[12] or within an hour with between-run CV ranging from 1.34% to 5.89% with the Vertical Auto Profiling method,[16] while HPLC methods can do it in 30 min with CV ranging from 0.3% to 4.5%.[17] NMR measurement (1D NOESY) reduces run time to 16 min (4 min for acquisition and 12 min for sample exchange, including temperature equilibration, autotuning, and matching) with CV 0−10% in low molecular weight metabolites.[18]

Therefore, determination of LPD from NMR spectra is desirable. Previous studies have successfully achieved this by extracting relevant information from the complex methylene (CH_2) and methyl (CH_3) signals in the 1.4−0.6 ppm region of the NMR spectra.[2,9,19] So far most large-scale studies on variation of LPDs were carried out at a single NMR center. In order to allow for distributed analysis at multiple NMR laboratories, good interlaboratory reproducibility is a prerequisite. Previous studies have addressed this issue on low molecular weight metabolites and large biomolecules,[6,18,20,21] but to the best of our knowledge, there is no study that focuses on interlaboratory variation and reproducibility in such large entities (supramolecular complexes) as lipoprotein particles.

The present study focuses on the implementation of a protocol and methodology that represents the latest technological development in NMR spectroscopy for a large-scale, interlaboratory, quantitative, reproducible, and high-throughput phenotyping of human blood plasma and serum with the aim to reliably predict lipoprotein particle distributions using NMR measurements from different laboratories. This NMR metabolomics protocol largely relies on controlled sample preparation, sample handling, data acquisition, and data processing using standardization of parameters. The protocol includes several control levels, including measurement temperature control within 0.1 K and spectral correction using ERETIC (Electronic REference To access In vivo Concentrations) signal equivalent to 10 mM proton. The standardized NMR protocol was applied in a ring test performed across three different laboratories in Europe for both plasma and serum samples obtained from the same individuals at the same time (Figure S1 of the Supporting Information). The ring test study enabled evaluation of the developed protocol for the quality and reproducibility of the data from three different laboratories to estimate several clinically important parameters of blood samples, including total cholesterol content in HDL and LDL. The NMR spectra from the three laboratories was investigated and scrutinized in terms of (1) spectral differences, (2) differences in the LPD predictions obtained using an existing prediction model,[9] and (3) agreement of these predictions with experimentally obtained standard clinical parameters.

MATERIALS AND METHODS

Experimental Design. Fasting plasma and serum samples of 30 adults were investigated at three different laboratories: University of Copenhagen (Frederiksberg, Denmark, labeled KU), Bruker BioSpin (Rheinstetten, Germany, labeled BR), and Unilever (Vlaardingen, Netherlands, labeled UL). After an overnight fasting (>10 h), blood samples were drawn and blood plasma and serum were separated. Aliquot fractions of blood plasma and serum were kept in the freezer (−80 °C) until shipped to the three laboratories in dry ice for 1D ^{1}H NMR analysis. Clinical measurements were taken for each participant.

Participants. Apparently healthy participants of both genders were selected based on the following inclusion criteria: >20 years old, body mass index (kg/m^2) between 18.5 and 40. Pregnant or lactating women (up to 6 weeks prior to study start), as well as subjects diagnosed with any form of cardiovascular disease or diabetes, reporting chronic gastrointestinal disorders, receiving antibiotic treatment within 3 months of starting the study, or using pre- or probiotic supplements within 1 month of starting the study, were not considered suitable candidates. All subjects were recruited in Copenhagen, via press announcements and online recruitment homepages. Elderly participants were also recruited via specific journals and social media platforms. All participants provided a written consent to participate in the study. The study was conducted at the Department of Nutrition, Exercise and Sports at the University of Copenhagen, Denmark, and was approved by the Research Ethics Committees of the Capital Region of Denmark in accordance with the Helsinki declaration (H-15008313) and the Danish Data Protection Agency (2013-54-0522).

NMR Sample Preparations. Plasma and serum samples were stored in cryovials at −80 °C until measurement day. Prior to preparation, they were thawed at room temperature for approximately half an hour. Respective aliquots of 350 μL were carefully mixed with equal amounts of phosphate buffer solution[22] in cryovials, and then transferred to 5 mm SampleJet tubes (Bruker BioSpin).

NMR Data Acquisition and Preprocessing. Samples were measured using 600 MHz NMR spectrometers from

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Anal. Chem. 2017, 89, 8004−8012

Bruker BioSpin equipped with an automated sample changer SampleJet (Bruker BioSpin) with sample cooling and preheating station, a 5 mm inverse probe with *z*-gradient and automated tuning and matching and cooling unit BCU-05 or BCU-I (Bruker BioSpin). The exact spectrometer and probes used in the ring test are listed in Table S1 of the Supporting Information. TopSpin 3.5 PL2 (Bruker BioSpin) was used for acquisition and processing. Automation of the overall measurement procedure was controlled by ICON NMR (Bruker BioSpin).

For NMR experiments the standard operational procedures (SOP) described in Dona et al. were applied.[6] Before measurement, each sample was kept for 5 min inside the NMR probe head in order to achieve temperature equilibration at 310 K with deviation <0.1 K. Then, automated tuning and matching, automated locking, automated shimming using TOPSHIM, automated optimization of the lock phase, and automated determination of the 90° hard pulse was applied to optimize the NMR experimental conditions. After this procedure, a one-dimensional ^{1}H NMR spectrum was acquired using a standard one-dimensional ^{1}H NMR pulse sequence with suppression of the water peak (Bruker pulse program library noesygppr1d), i.e., RD−P(90°)−4 μs−P(90°)−t_m−P(90°) acquisition of the free induction decay (FID). The relaxation delay (RD) and mixing time t_m, were set to 4 s and 10 ms, respectively. Low-power continuous wave irradiation (CW) for saturation of the water resonance at rf field strengths of 25 Hz was applied during RD and t_m for presaturation. Prior to the first and the last pulse, a *z*-gradient was applied. After application of 4 dummy scans, 32 free induction decays (FIDs) were collected into 98 304 (96k) data points using a spectral width of 30 ppm (18 028.846 Hz). The receiver gain was kept constant (RG = 90.5) for all samples. FIDs were multiplied with an exponential function corresponding to a line broadening of 0.3 Hz prior to Fourier transformation and fully automated phasing and baseline correction via the Bruker standard automation program APK0.NOE. Quantitative calibration of the spectra was ensured using the PULCON principle.[23]

Raw NMR spectra were scaled to the ERETIC signal[24] at 15 ppm, equivalent to 10 mM protons, and then aligned to the alanine doublet in the region of 1.494−1.507 ppm using icoshift[25] with an offset modification (icoshift ver. 3.0; Matlab source code is freely available for download at www.models.life.ku.dk/algorithms), using command: [xCS,ints,ind,target] = icoshift('average',data,'1.507−1.494','b',[2 1 0 20 1 1],ppm_scale).

Standard Clinical Parameters. Analyses of plasma and serum total cholesterol, HDL-C, LDL-C, and total triglycerides were done using the automated, enzymatic, colorimetric assay on the ABX Pentra 400 chemistry analyzer (ABX Pentra, Horiba ABX, Montpellier, France). The CV% for these analyses was between 1.2% and 3.1%.

Sample Selection. One of the 30 subjects included in the ring test study was excluded from the analysis due to lack of the ERETIC signal, and another subject was excluded because no plasma standard clinical measurements were made. This resulted in plasma and serum NMR spectra and clinical measurements for 28 individuals. Thus, a total of 168 1D ^{1}H NMR spectra were included in this study (28 subjects × 2 sample types × 3 laboratories).

Partial Least Squares Model Transfer for LPD Estimation. The model used in this study to determine lipoprotein distributions is the one presented in Mihaleva et al.[9] This partial least squares (PLS) model, which was calibrated on HPLC data,[15] predicts cholesterol and triglyceride concentrations in 3 main lipoprotein particle classes (HDL, VLDL, LDL) and 13 subclasses (VLDL03, VLDL04, VLDL05, VLDL06, VLDL07, LDL08, LDL09, LDL10, LDL11, HDL15, HDL16, HDL17, HDL18) from serum NMR spectra.

In order to successfully apply this PLS model, the scaled and aligned ring test data were further processed (model transfer). To remove chemical shift differences between the ring test NMR data and the mean 1D ^{1}H NMR spectrum of the model data,[9] the ring test data was further aligned to the alanine doublet of the model data using the mean spectrum as a target with icoshift[25] as described earlier. From these realigned spectra, only the 1.4−0.6 ppm region was considered for the data analysis, as this region contains the methylene and methyl bands that allow for the prediction of cholesterol and triglycerides concentrations with the PLS model from Mihaleva et al.[9] Given that for this region we had 3492 data points and the model comprises 1746 data points, we used a binning size of 2. The data was then rescaled to the mean of the data used to build the model of Mihaleva et al.[9] in order to get intensities of similar magnitude; for this normalization, a scaling factor was determined (Figure S2 of the Supporting Information). Because the PLS model used in this study was built on serum data, we calculated the scaling factor c using the serum ring test NMR spectra (eq 1)

$$c = \frac{\frac{1}{n}\sum_{i=1}^{n}\sum_{j=1}^{p} a_{ij}}{\frac{1}{m}\sum_{i=1}^{m}\sum_{j=1}^{p} b_{ij}} \quad (1)$$

where matrix **A** contains the training set intensities of Mihaleva et al.,[9] matrix **B** contains the ring test serum spectral data, n is the number of samples of the Mihaleva et al.[9] training set (n = 190), m is the number of samples in the serum data set (m = 84), and p is the number of data points considered (p = 1746).

Plasma and serum data were then multiplied by this scaling factor (Figure S2 of the Supporting Information). Cholesterol predictions were obtained on the region 1.04−0.6 ppm (971 chemical shifts) of the spectra, corresponding to the methyl signal. Total triglyceride predictions were obtained using the region 1.4−0.6 ppm of the spectra (1746 chemical shifts), corresponding to the methyl and methylene signals.[9]

Data Analysis. The results are structured according to three different levels (Figure 1). Level 1 describes differences between laboratories (BR/KU/UL) and between sample

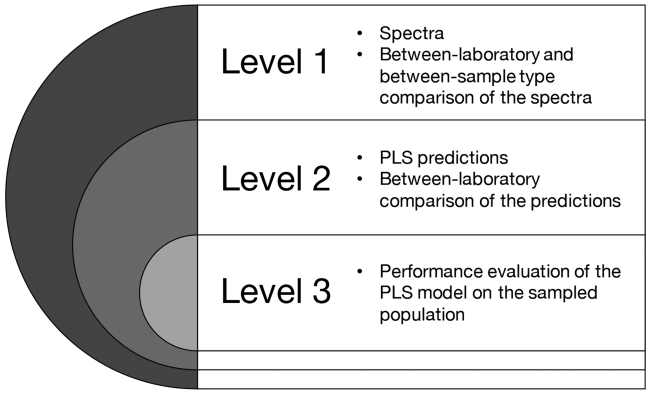

Figure 1. Schematic of the different levels of the data analysis.

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Anal. Chem. 2017, 89, 8004−8012

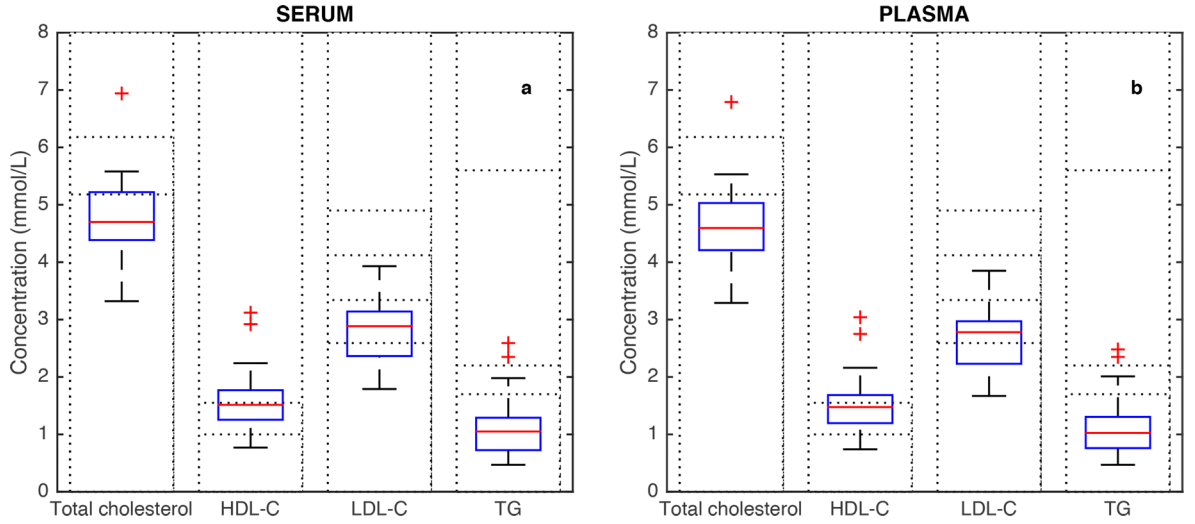

Figure 2. Total cholesterol, HDL-C, LDL-C, and total triglyceride (TG) concentration distributions of the (a) serum and (b) plasma samples from 28 subjects. The dotted lines indicate the different levels in the serum lipid classification of the National Cholesterol Education Program (NCEP) Adult Treatment Panel III (for more details see Table S2 of the Supporting Information).

types (plasma/serum) in the NMR spectra. Level 2 describes the differences between laboratories on their ability to predict LPDs from the PLS model presented in Mihaleva et al.[9] At level 3, the performance of the PLS model is evaluated by comparing the lipid predictions with measured standard lipid parameters.

Level 1, Spectral Differences: Between-Sample Type and between-Laboratory Comparisons. Since the ultimate target of this paper is to assess the reproducibility of NMR data acquisition for LPD determination across laboratories, only the relevant part of the spectra that includes the methylene and methyl signals is included in the calculations: 1.4−0.6 ppm. Principal component analysis (PCA) and ANOVA-simultaneous component analysis (ASCA)[26] were performed with a view to understanding the variation in the data. Unlike PCA, where the three different sources of variation (subject, sample type, laboratory) cannot be distinguished, ASCA allows for the partitioning of the variance in the data into different factors. This way, the contribution of the different sources of variation can be properly assessed. Additionally, a dissimilarity measure between data obtained at the different laboratories and between data obtained from the different sample types (plasma and serum) was calculated as indicated in eq 2:

$$r = \sqrt{\frac{\mathrm{SS}(\mathbf{A}-\mathbf{B})}{\mathrm{SS}(\mathbf{A})+\mathrm{SS}(\mathbf{B})}} \qquad 0 \le r \le 1 \tag{2}$$

where SS indicates sum of squares and **A** and **B** are the spectral subsets to be compared (subsets from different laboratories for the between-laboratory comparison and subsets from the two different sample types for the between-sample type comparison). A dissimilarity value of $r = 0$ indicates maximum similarity and $r = 1$ maximum dissimilarity. For derivation, see eq S1 of the Supporting Information.

Level 2, Lipoprotein Particle Distribution Prediction: Between-Laboratory Comparisons. Lipoprotein particle distributions were predicted from the NMR data using the PLS model presented in Mihaleva et al.[9] Because this model was built on HPLC data, cholesterol and triglyceride predictions of 3 main classes (HDL, VLDL, LDL) and 13 subclasses (VLDL03, VLDL04, VLDL05, VLDL06, VLDL07, LDL08, LDL09, LDL10, LDL11, HDL15, HDL16, HDL17, HDL18) were obtained for the ring test subjects.

In order to determine the contribution of the three factors (subject, sample type, and laboratory) to the total variation in the cholesterol and total triglyceride predictions, a linear model was fitted on the predictions of all subclasses and ANOVA was performed. For the determination of between-laboratory variation per sample type (plasma/serum) absolute root-mean-square error (RMSE) was calculated on the prediction of all (sub)classes (eq 3, where y_1 and y_2 are the predictions of two different laboratories).

$$\mathrm{RMSE} = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(y_{1_i} - y_{2_i})^2} \tag{3}$$

Level 3, Model Performance on the Prediction of Standard Lipid Parameters. HDL class is the most suitable one to assess the model performance on the ring test data, given that it is very well-defined, allowing cholesterol measurements for this class to be compared across different techniques (i.e., standard clinical measurements and HPLC measurements). At this level, the model performance was assessed per laboratory, for both plasma and serum samples, using the RMSE and Pearson correlation coefficient of predicted and measured values of HDL-C and additionally also of LDL-C (eq 3, where y_1 and y_2 are the predicted and clinical parameters, respectively).

RESULTS AND DISCUSSION

Characterization of the Ring Test Population. The subjects participating in the ring test were classified according to their lipid levels as described by the National Cholesterol Education Program (NCEP) Adult Treatment Panel III guidelines (2002) based on serum values (Figure 2, Table S2 of the Supporting Information).[27] Even though the lipid panels of plasma and serum samples from the same subjects are very similar (with a correlation of 0.9874, 0.9973, 0.9919, and 0.9960 for total cholesterol, HDL-C, LDL-C, and total triglycerides, respectively), small lipid measurement differences lead in some cases to different classification (Table S2 of the Supporting Information). The majority of the subjects sampled for the ring test had average or close to average lipid values

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Table 1. Description of the Classical Lipid Panel (mmol/L) of the 28 Ring Test Plasma and Serum Samples and of 189 Mihaleva Training Samples (Ref 28)[a]

lipid	cohort	min	max	mean	median	SD
total cholesterol	ring test (plasma)	3.29	6.79	4.66	4.60	0.69
	ring test (serum)	3.32	6.94	4.78	4.70	0.71
	Mihaleva (training)	3.10	8.10	6.26	6.30	0.87
HDL-C	ring test (plasma)	0.74	3.04	1.52	1.48	0.50
	ring test (serum)	0.77	3.12	1.57	1.52	0.52
	Mihaleva (training)	0.80	3.00	1.64	1.60	0.39
LDL-C	ring test (plasma)	1.67	3.85	2.65	2.78	0.54
	ring test (serum)	1.79	3.93	2.80	2.89	0.56
	Mihaleva (training)	1.30	5.90	3.78	3.80	0.83
total triglycerides	ring test (plasma)	0.47	2.48	1.13	1.03	0.50
	ring test (serum)	0.47	2.59	1.11	1.05	0.52
	Mihaleva (training)	0.47	2.04	1.08	1.00	0.38

[a]Clinical data for one of the original 190 Mihaleva training samples was not available.

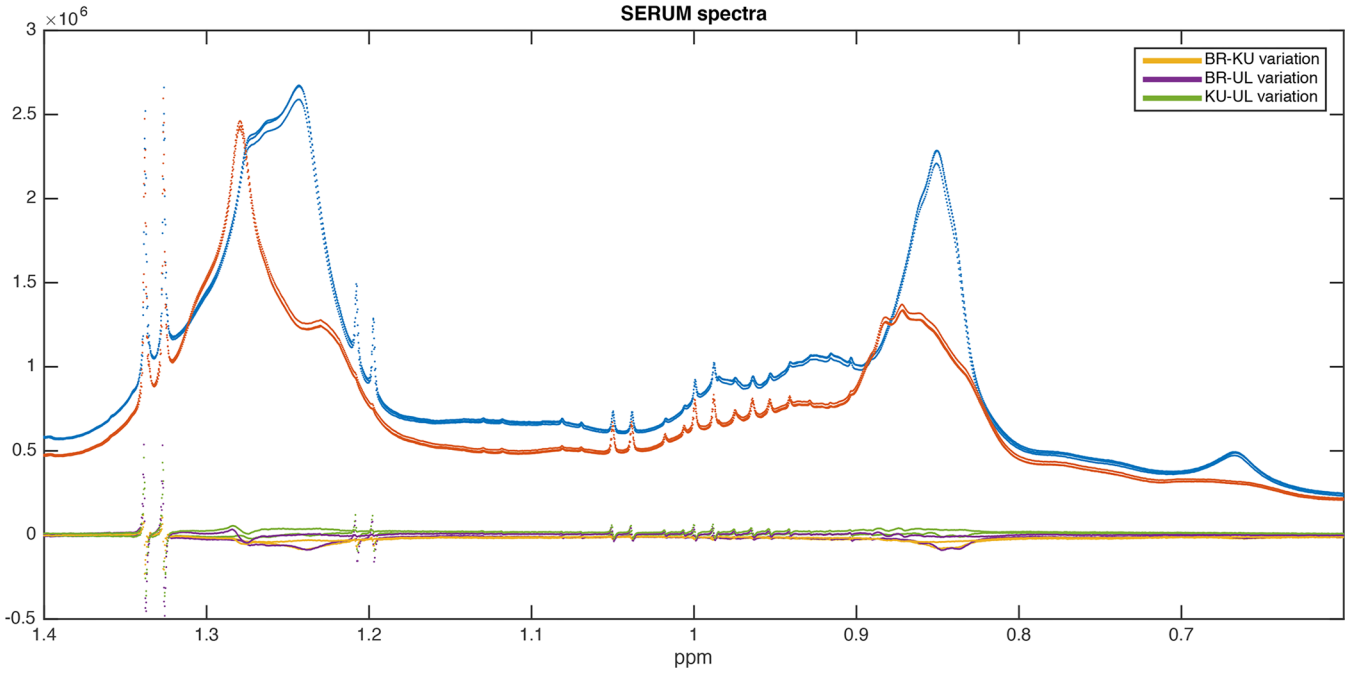

Figure 3. Serum spectra of two representative subjects obtained in three different laboratories (BR, KU, UL). NMR data of the same subject are very similar between laboratories, resulting in three same-colored (blue, orange) overlapping lines. Also shown are the differences between NMR spectra across laboratories for the two individuals (yellow, purple, and green lines).

(Figure 2, Table S2 of the Supporting Information). Since most of the ring test samples fall within the ranges of the cohort in which the model from Mihaleva et al.[9] was built,[28] we determined that the model was applicable to the ring test cohort (Table 1).

Structure of the Data Analysis. The results are structured according to the different levels specified in Figure 1. At level 1, differences between laboratory (BR/KU/UL) and between sample type (plasma/serum) in the spectra are analyzed. At level 2, an existing PLS model to predict lipoprotein subclass from serum NMR spectra[9] is used to obtain lipid predictions from all spectra; differences between laboratories in the predictions are studied. At level 3, the performance of the PLS model on the ring test population is evaluated by comparing the PLS predictions with the classical lipid panel measurements.

Level 1: Spectral Differences. In this study, the sources of variation present in the NMR data (1.4−0.6 ppm, 1746 data points) are (i) the subjects, (ii) the type of sample (plasma and serum), and (iii) the laboratory (BR, KU, UL). PCA of all spectra (Figure S3 of the Supporting Information) and PCA of the plasma and serum spectra separately (Figure S4 of the Supporting Information) indicate that between-subject variation dominates over between-sample type (plasma/serum) variation, which in turn dominates over the between-laboratory variation. Figure 3 points out how small the latter is and gives an idea of the level of reproducibility of NMR measurements across laboratories.

Given the multifactorial nature of the data, PCA fails to provide a good interpretation of the variation in the data. In order to improve this, ASCA was applied, as this type of analysis allows for a good separation of all sources of variation on the mean-centered spectral data (Figure S5 of the Supporting Information).

The ASCA analysis determined that the between-subject variation represents 97.99% of the overall explained variation in

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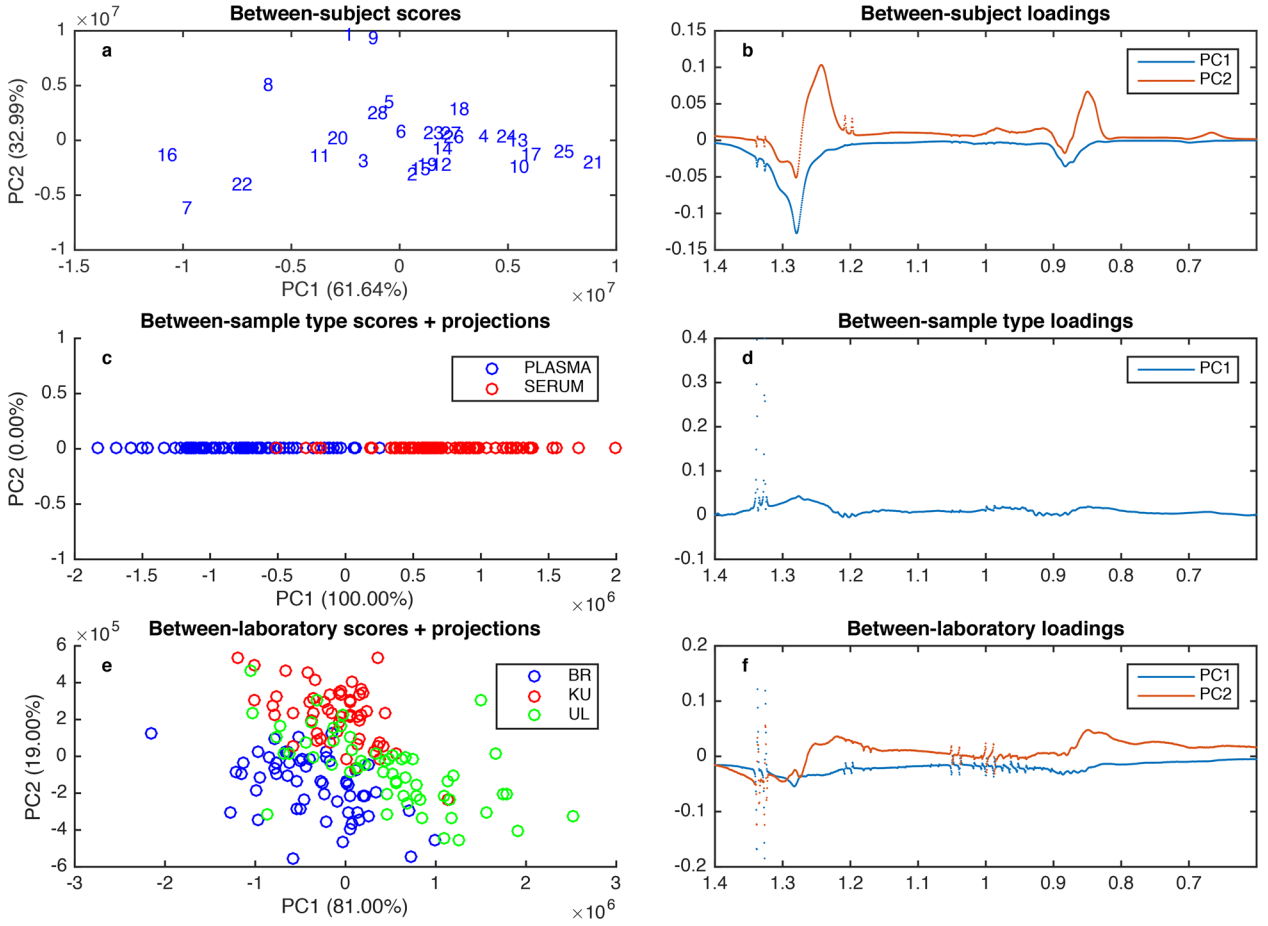

Figure 4. (a) Scores plot and (b) loadings plot of the factor *subject*; (c) scores plot with the residuals of all samples projected and (d) loadings plot of the factor *sample type*; (e) scores plot with the residuals of all samples projected and (f) loadings plot of the factor *laboratory* of the ASCA analysis on the NMR spectral data.

the data (Figure 4, parts a and b). The between-sample type and between-laboratory variations of the spectra were already visible in the PCA score plots (Figures S3 and S4 of the Supporting Information), but a much clearer sample grouping is revealed in the score plots of the factor subject from the ASCA analysis (Figure 4a). The between-sample type variation represents 1.62% of the overall explained spectral variation (Figure 4, parts c and d), while the between-laboratory variation represents 0.39% of the total explained variation (Figure 4, parts e and f). Tables 2 and 3 also confirm that between-sample type variation is larger than between-laboratory variation, as the dissimilarity values in the between-sample type comparison are larger than those found in the between-lab comparison.

Table 2. Dissimilarity between the Spectra from the Three Laboratories (BR, KU, UL), for Both the Plasma and Serum Subsets[a]

plasma	BR	KU	UL	serum	BR	KU	UL
BR	0	0.015	0.030	BR	0	0.016	0.032
KU	0.015	0	0.025	KU	0.016	0	0.026
UL	0.030	0.025	0	UL	0.032	0.026	0

[a]All dissimilarity values are smaller than 0.056, which corresponds to the dissimilarity between the data sets here used and the same data set shifted 0.001 ppm to the right (misalignment). All dissimilarity values are also smaller than 0.0412, which corresponds to the dissimilarity between the ring test data (**A** = **X**) and the same data with a 6% addition (**B** = 1.06**A**).

Table 3. Dissimilarity between the Serum and the Plasma Spectra for Each of the Three Laboratories (BR, KU, UL)[a]

BR	KU	UL
0.035	0.037	0.038

[a]All dissimilarity values are smaller than 0.056, which corresponds to the dissimilarity between the data sets here used and the same data set shifted 0.001 ppm to the right (misalignment). All dissimilarity values are also smaller than 0.0412, which corresponds to the dissimilarity between the ring test data (**A** = **X**) and the same data with a 6% addition (**B** = 1.06**A**).

A PCA performed on the matrix corresponding to the factor laboratory results in a PC1 (explaining 81% of the variation) that revolves mostly around the deviation of the UL spectra. Inspection of the corresponding PC1 loadings shows that this deviation could be related to slightly lower intensities of the UL measurements. PC2 of the between-laboratory variation, which explains 19% of the between-laboratory variation, clearly separates the spectra obtained at the KU from those obtained at BR, with the UL spectra placed in between. Inspection of the corresponding PC2 loadings show that this variation is related to a shift (or peak shape) in the lactate doublet at 1.33 ppm and presumably a small shift in the broad methylene and methyl signals. Overall, it is observed that the BR and KU spectra are more similar to each other and that the UL spectra exhibits

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Anal. Chem. 2017, 89, 8004−8012

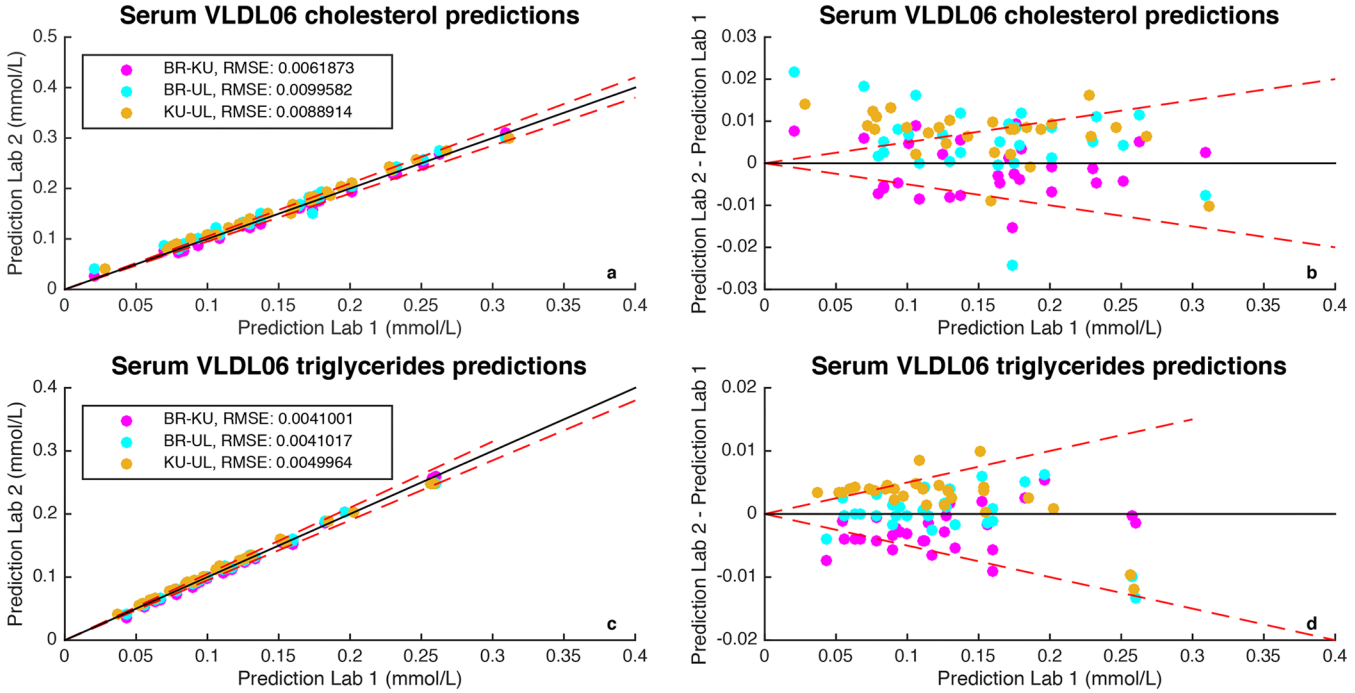

Figure 5. Between-lab differences in serum cholesterol and triglycerides predictions for the VLDL06 subclass obtained using the Mihaleva-derived PLS model. BR vs KU, BR vs UL, and UL vs KU (a and b) cholesterol and (c and d) triglycerides serum predictions. (a and c) On the *x* axis, the value of the prediction of the first laboratory; on the *y* axis, the value of the prediction of the second laboratory. (b and d) Zoom-in of the differences between laboratories: on the *x* axis, the value of the prediction of the first laboratory; on the *y* axis, the difference between the prediction of the second laboratory and the prediction of the first one. The black line is drawn where predictions are equal; the red dashed lines indicate a deviation of 5%.

deviation from these (Figure 4e, Tables 2 and 3). However, it should be emphasized that this data analysis scrutinizes the variation between laboratories, which only represents 0.39% of the overall systematic variation and is already smaller than the 1% acceptable intralab spectroscopic variation suggested by Dona et al.[6] Several factors might be playing a role in the small deviations of the UL spectra, including (i) the slightly different NMR probe used at UL (Inverse Cryo TCI) and (ii) tiny fluctuations in the air conditioning experienced at the UL lab, which can lead to unstable room temperature and thus to small peak broadening in some spectra.

The between-sample type and between-laboratory variations are mostly due to the lactate doublet in the 1.345−1.32 ppm region (Figure 4, parts d and f). If the lactate doublet is removed, the percentage of the variation explained by sample type drops to 0.53%, while the percentage of the variation explained by the laboratory is not largely reduced (0.32%) and subject explains 99.15% of the variation. These results seem to indicate that sample type variation is largely related to minor pH differences.

All in all, both the between-sample type variation and the between-lab variation in the ring test NMR spectra were determined to be low. The fact that between-lab variation is so small indicates that the NMR acquisition protocol here described is suitable for cross-cohort studies by allowing direct interindividual comparison of NMR data obtained in different laboratories.

Level 2: Lipoprotein Particle Distribution Prediction. In the previous section (level 1) it was shown that the spectra from the three different laboratories are very similar. In this section, the effect of the small spectral differences in the NMR data obtained at the three different laboratories on the prediction of cholesterol and triglyceride (TG) concentrations of lipoprotein subclasses will be investigated. In order to do so, the model derived from Mihaleva et al.,[9] built on an apparently healthy, hypercholesterolemic human population, is here applied to estimate cholesterol and triglyceride content for a total of 16 lipoprotein particle classes from the NMR data. Subsequently, these predictions are analyzed in terms of between-laboratory differences.

The lipid content of the samples of 28 subjects was predicted from the serum and plasma NMR spectra of the three laboratories separately. In Figure 5, the between-laboratory differences in cholesterol and triglycerides (TG) serum predictions in the VLDL06 subclass are depicted: BR versus KU, BR versus UL, and UL versus KU. This subclass (VLDL06) is representative of the general trend in all subclasses (Figure S6 of the Supporting Information). RMSE of the predictions obtained using data from the three different laboratories was calculated to compare the predicted cholesterol and TG concentrations (Figure 5). The RMSE of the comparisons UL−BR and UL−KU of cholesterol predictions is slightly larger than the RMSE of the comparison BR−KU. For the TG predictions, all lab comparisons result in similar RMSE. The majority of data points lie between the lines that indicate a ±5% deviation. Some data points fall outside of the lines; however, it should be noted that the majority of these points correspond to very low cholesterol and triglyceride concentration predictions. Thus, the relative deviation might be larger than 5%, but the absolute deviation (calculated as RMSE) remains very small. Despite the fact that the model derived from Mihaleva et al.[9] is built on serum data, it is observed that the cholesterol and TG predictions from plasma

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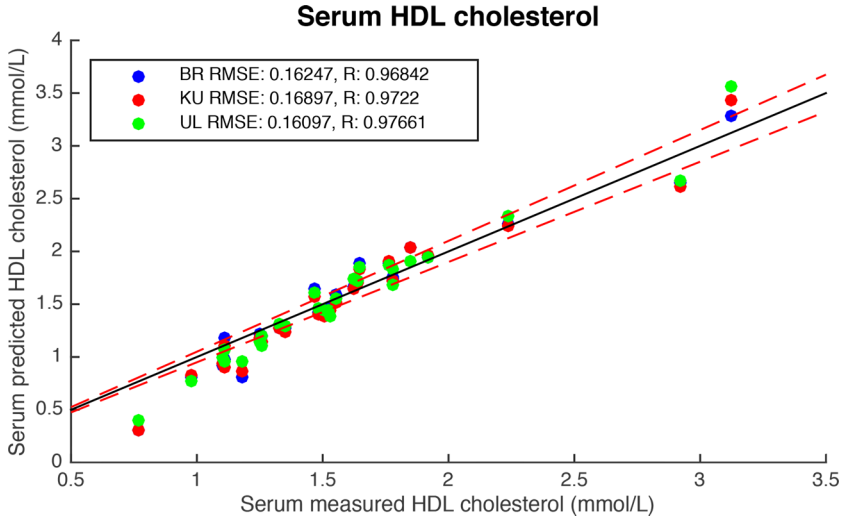

Figure 6. HDL-C predictions of the PLS model on serum spectra from the three laboratories. The black line is drawn where prediction and measurement are equal; the red dashed lines indicate plus or minus 5%.

NMR data of all three laboratories differ in a similar way (Figure S7 of the Supporting Information).

A linear model was fit separately on the cholesterol and triglycerides predictions for all subclasses. Using this model, subject explains 99.16%, sample type 0.73%, and laboratory 0.11% of the total variation of the cholesterol predictions explained by these three factors. In the case of the triglyceride predictions, subject explains 99.44% of the variation, sample type 0.48%, and laboratory 0.08% of the variation. According to these values, both the sample type and the laboratory variation in the predictions are reduced when compared to the original NMR spectral differences given by the ASCA analysis (97.99% for subject, 1.62% for sample type, 0.39% for laboratory). The reduction in the sample type and lab variation in LPD predictions is most likely due to the fact that the regions of the spectra that contribute the most to the NMR variation do not have a determinant weight (in terms of regression coefficients) on the PLS predictions.

Summarizing, the already low NMR between-lab variation translates into even lower between-lab differences in lipid profile predictions. Thus, implementation of this NMR protocol would enable LPD phenotyping of large cohorts across different laboratories.

Level 3: Model Performance on the Prediction of Standard Lipid Parameters. In previous sections, it was shown that differences in spectra from different laboratories and different sample types (plasma/serum) are very small (level 1), translating into even smaller differences in the prediction of lipoprotein distribution (level 2). In this section (level 3), the performance of the PLS model on this cohort is evaluated by comparing its predictions to the experimental measurements (classical lipid panel). Figure 6 shows the comparison of the HDL-C serum predictions to the serum HDL-C clinical measurements. The results show that the predictions made on NMR data from different laboratories for the same subject are very similar, even when the predicted values differ from the measured values. The RMSE values, again very close to each other between laboratories, are slightly larger than the one reported for the Mihaleva test set HDL-C prediction (0.0679).[9] The Pearson correlation coefficients are slightly smaller than the Pearson correlation coefficient reported for the HDL-C class in the Mihaleva test set (0.99).[9] In spite of the fact that this PLS model was built with HPLC data as response data and on a different cohort, these results show that the model allows for a good prediction of HDL-C clinical measurements on the population sampled in this study. The residuals of the fit of PLS predictions and HDL-C clinical values were further investigated (Figure S8 of the Supporting Information). This analysis corroborated the good fit of the model for average values and the presence of larger errors for the subjects with more deviating HDL-C values. The latter were not contained within the range of the clinical HDL-C values of the training set in the Mihaleva model (Table 1) and thus had to be extrapolated by the model. Despite the fact that the model derived from Mihaleva et al.[9] is built on serum data, it is observed that the model also performs well on plasma data (Figure S9 of the Supporting Information). Results for the LDL-C class are shown in Figure S10 of the Supporting Information. For this class, the predictions do not correlate with the clinical measurements as well as for the HDL-C class, which might be a combination of an initial lower performance of the model on the LDL class, differences in response data (clinical vs HPLC) and differences in distributions across cohorts (Table 1). However, the predictions of the three different laboratories are still very close.

CONCLUSIONS

The present study evaluated the reproducibility of a standardized protocol and methodology for the acquisition of NMR data on blood plasma and serum samples at different laboratories prior to the estimation of clinically relevant lipid parameters of blood.

Analysis of this ring test NMR data showed that the variation between plasma and serum and the interlaboratory variation are small when the measurement conditions are met. In fact, the interlaboratory variation in the NMR spectral data was found to be smaller than the maximum acceptable intralaboratory variation on the quality control samples.[6] In order to determine the effect of the small spectral differences upon lipoprotein predictions, an existing model[9] for the prediction of lipoprotein parameters was applied to the recorded spectra. The predictions from this model proved to be very similar (even more than in the original NMR spectra) between laboratories in both serum and plasma samples. The latter fact confirmed

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that the reproducibility between different laboratories is good enough for the lipoprotein profile predictions to be exchangeable.

The fact that both the population sampled in the Mihaleva et al.[9] study and the population studied in the present ring test were apparently healthy may be the reason why it was possible to successfully apply this existing lipoprotein model to our data after a model transfer. A future challenge will be to build a model for LPD prediction that covers populations comprising a wide range of phenotypes, i.e., lipoprotein particle distributions.

With the successful implementation of this standardized protocol for obtaining reproducible data between different laboratories, a step is taken toward bringing NMR measurement of lipoprotein distributions into biomarker development and diagnostics. By using an NMR-based approach to predict LPDs, research and diagnostics can benefit from the added value of lipid profile in multiple lipoprotein particle subclasses while reducing the need for less efficient methods such as ultracentrifugation or HPLC. Implementation of this protocol for reproducible acquisition of NMR data will potentially allow LPD phenotyping of large cohorts across different laboratories in future metastudies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.7b01329.

Experimental design of the ring test study; Spectrometer and probes used; Mean spectra of the ring test and Mihaleva cohorts; Lipid distribution of the ring test and Mihaleva cohorts; PCA score plots; Data partitioning in the ASCA analysis; Between-laboratory differences in serum and plasma cholesterol and triglycerides predictions using the Mihaleva-derived PLS model; Ring test HDL-C residual plot; HDL-C predictions of the PLS model on plasma spectra from the three laboratories, and LDL-C predictions of the PLS model on serum and plasma spectra from the three laboratories (PDF)

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Notes

The authors declare no competing financial interest.

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